

Quantikine[®]

Human Clusterin Immunoassay

Catalog Number DCLU00

For the quantitative determination of human Clusterin concentrations in cell culture supernates, serum, plasma, urine, and saliva.

This package insert must be read in its entirety before using this product.

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**FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

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INTRODUCTION

Clusterin [also known as Apolipoprotein J, Sulfated Glycoprotein 2 (SGP-2), TRPM-2, and SP-40], is a secreted multifunctional protein that was named for its ability to induce cellular clustering. It binds a wide range of molecules and may function as a chaperone of misfolded extracellular proteins. It also participates in the control of cell proliferation, apoptosis, and carcinogenesis (1, 2). Clusterin is predominantly expressed in adult testis, ovary, adrenal gland, liver, heart, brain, and in many epithelial tissues during embryonic development (3). Human Clusterin is synthesized as a precursor that contains two coiled coil domains, three nuclear localization signals (NLS), and one heparin binding domain (4 - 6). Intracellular cleavages of the precursor remove the signal peptide and generate comparably sized α and β chains which are secreted as an approximately 80 kDa N-glycosylated and disulfide-linked heterodimer (7 - 9). Mature human Clusterin shares a 77% amino acid sequence identity with mouse and rat Clusterin.

High $\mu\text{g/mL}$ concentrations of Clusterin circulate predominantly as a component of high density lipoprotein particles, and these are internalized and degraded through interactions with LRP-2/Megalin (10, 11). The ability of Clusterin to bind and neutralize non-oxidatively modified LDL reduces cytotoxicity in atherosclerotic plaques (12). The chaperone function of Clusterin helps to reduce the accumulation of β -amyloid fibrils and damage due to amyloid plaques in Alzheimer's disease (13). An alternately spliced 50 kDa isoform of human Clusterin (nCLU) remains intracellular and is neither glycosylated nor cleaved into α and β chains (5, 14). Cellular exposure to ionizing radiation promotes the translocation of nCLU to the nucleus where it interacts with Ku70 and promotes apoptosis (5, 14). This function contrasts with the cytoprotective effect of secreted Clusterin (15). During tumor progression, nCLU is downregulated while the secreted form is upregulated and may be aberrantly glycosylated (14, 16, 17). Increased circulating levels of Clusterin enhance tumor aggressiveness by inhibiting apoptosis and by promoting the epithelial to mesenchymal transition (18 - 20).

The Quantikine Human Clusterin Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Clusterin in cell culture supernates, serum, plasma, urine, and saliva. It contains NS0-expressed recombinant human Clusterin and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Clusterin showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that the Quantikine Human Clusterin kit can be used to determine relative mass values for naturally occurring human Clusterin.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Clusterin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Clusterin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for Clusterin is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Clusterin bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED

Clusterin Microplate (Part 893769) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against Clusterin.

Clusterin Conjugate (Part 893770) - 21 mL of monoclonal antibody against Clusterin conjugated to horseradish peroxidase with preservatives.

Clusterin Standard (Part 893771) - 1 µg of recombinant human Clusterin in a buffered protein base with preservatives; lyophilized.

Assay Diluent RD1-19 (Part 895467) - 11 mL of a buffered protein base with preservatives.

Calibrator Diluent RD5T (Part 895175) - 4 vials (21 mL/vial) of a buffered protein base with preservatives.

Wash Buffer Concentrate (Part 895003) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.

Color Reagent A (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895032) - 6 mL of 2 N sulfuric acid.

Plate Covers - 4 adhesive strips.

STORAGE

Unopened Kit	Store at 2 - 8° C. Do not use past kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Assay Diluent RD1-19	
	Calibrator Diluent RD5T	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Standard	Aliquot and store at $\leq -70^{\circ}$ C for up to 1 month.* Avoid repeated freeze-thaw cycles.
Microplate Wells	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*	

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Test tubes for dilution.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Human Clusterin Controls (optional; available from R&D Systems).

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

Clusterin is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma is not validated for use in this assay.*

Saliva - Collect saliva directly into a test tube, and centrifuge at 10,000 rpm for 10 minutes. Collect the aqueous layer. Assay immediately or aliquot and store samples at $\leq -80^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Note: *Do not use a saliva collector.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at $\leq -80^{\circ}$ C. Avoid repeated freeze-thaw cycles. For more information on collecting and handling urine specimens, refer to the National Committee for Clinical Laboratory Standards (NCCLS) publication GP16-T.

SAMPLE PREPARATION

Serum and plasma samples require a 2000-fold dilution. A suggested dilution is 25 μ L of sample + 975 μ L of Calibrator Diluent RD5T. Complete the 2000-fold dilution by adding 20 μ L of the diluted sample to 980 μ L of Calibrator Diluent RD5T.

Urine samples require at least a 4-fold dilution. A suggested 4-fold dilution is 50 μ L of sample + 150 μ L of Calibrator Diluent RD5T.

Saliva samples require at least a 2-fold dilution. A suggested 2-fold dilution is 100 μ L of sample + 100 μ L of Calibrator Diluent RD5T.

Cell culture supernate samples may require dilution.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

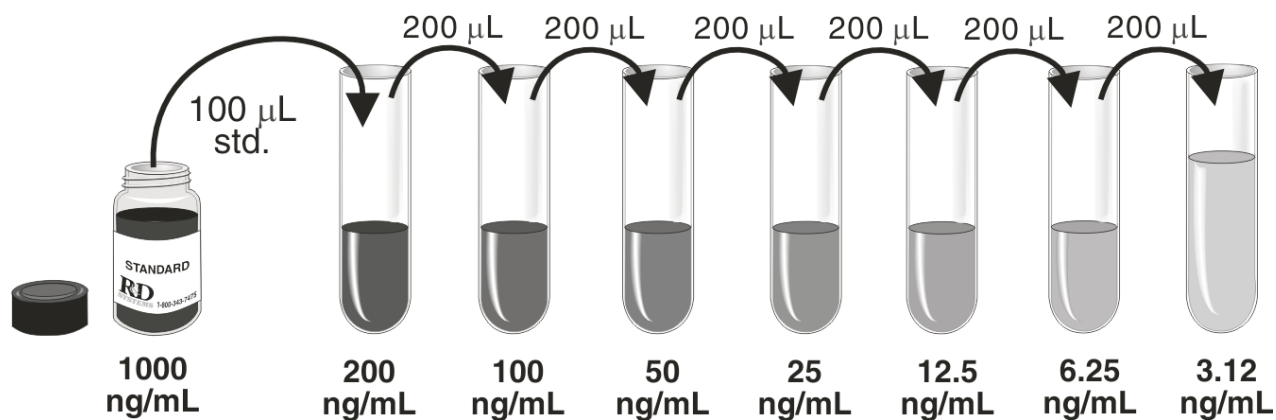
Note: High concentrations of Clusterin are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μ L of the resultant mixture is required per well.

Clusterin Standard - Reconstitute the Clusterin Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 1000 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 400 μ L of Calibrator Diluent RD5T into the 200 ng/mL tube. Pipette 200 μ L of Calibrator Diluent RD5T into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 200 ng/mL standard serves as the high standard. Calibrator Diluent RD5T serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

Note: *High concentrations of Clusterin are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 μL of Assay Diluent RD1-19 to each well.
4. Add 50 μL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 μL of Clusterin Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 μL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 μL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Most samples require dilution. See the Sample Preparation section.

ASSAY PROCEDURE SUMMARY

1. Prepare reagents, samples, and standards as instructed.



2. Add 100 μL of Assay Diluent RD1-19 to each well.



3. Add 50 μL of Standard, control, or sample* to each well. Incubate for 2 hours on the shaker at RT.



4. Aspirate and wash 4 times.



5. Add 200 μL of Conjugate to each well. Incubate for 2 hours on the shaker at RT.



6. Aspirate and wash 4 times.



7. Add 200 μL of Substrate Solution to each well. Incubate for 30 minutes **on the benchtop. Protect from light.**



8. Add 50 μL of Stop Solution to each well. Read at 450 nm within 30 minutes.
 λ correction 540 or 570 nm

*Most samples require dilution.

CALCULATION OF RESULTS

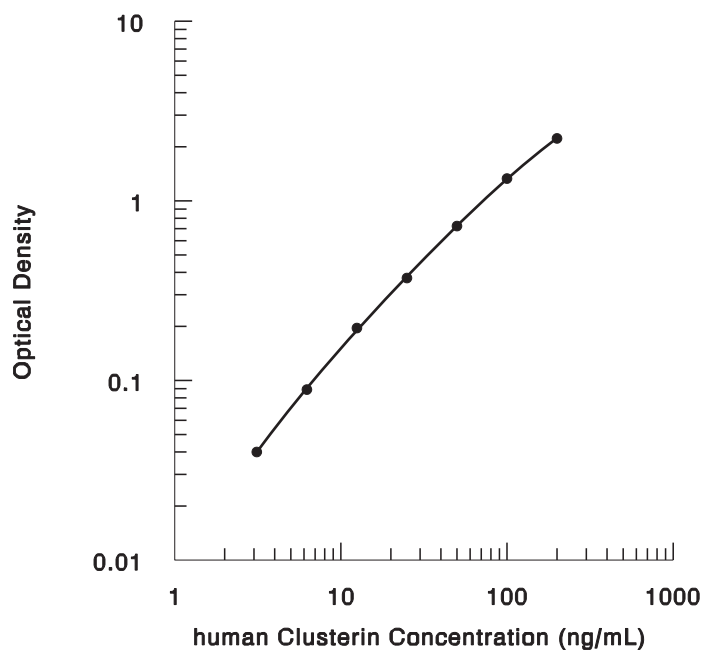
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the Clusterin concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



ng/mL	O.D.	Average	Corrected
0	0.006 0.006 0.045	0.006	—
3.12	0.047 0.094	0.046	0.040
6.25	0.096 0.201	0.095	0.089
12.5	0.202 0.377	0.202	0.196
25	0.378 0.727	0.378	0.372
50	0.732 1.312	0.730	0.724
100	1.363 2.230	1.338	1.332
200	2.238	2.234	2.228

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

PRECISION

Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in forty separate assays to assess inter-assay precision.

Sample	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	19.0	64.3	130	22.1	64.1	136
Standard deviation	0.7	2.4	4.5	1.6	5.4	9.3
CV (%)	3.4	3.7	3.5	7.2	8.4	6.8

RECOVERY

The recovery of Clusterin spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample	Average % Recovery	Range
Cell culture media (n=4)	107	102 - 110%

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of Clusterin were serially diluted with the Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates* (n=4)	Serum* (n=4)	Heparin plasma* (n=4)	EDTA plasma* (n=4)	Saliva* (n=4)	Urine* (n=4)
1:2	Average % of Expected	103	96	99	97	103	98
	Range (%)	99 - 110	94 - 98	98 - 99	93 - 101	100 - 106	95 - 101
1:4	Average % of Expected	98	94	97	94	102	91
	Range (%)	92 - 109	92 - 95	86 - 102	91 - 97	97 - 106	88 - 92
1:8	Average % of Expected	96	92	95	91	100	89
	Range (%)	89 - 107	89 - 95	85 - 100	87 - 95	89 - 107	83 - 94
1:16	Average % of Expected	92	90	92	89	97	89
	Range (%)	88 - 97	87 - 93	82 - 99	86 - 94	89 - 106	83 - 94

*Samples were diluted prior to assay.

SENSITIVITY

Fifty-seven assays were evaluated and the minimum detectable dose (MDD) of Clusterin ranged from 0.064 - 1.050 ng/mL. The mean MDD was 0.189 ng/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human Clusterin produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine - Samples from apparently healthy volunteers were evaluated for the presence of Clusterin in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (µg/mL)	Standard Deviation (µg/mL)	Range (µg/mL)
Serum (n=36)	266	42.8	195 - 378
Heparin plasma (n=36)	266	44.6	190 - 377
EDTA plasma (n=36)	261	52.0	185 - 436

Sample Type	Mean (ng/mL)	Standard Deviation (ng/mL)	Range (ng/mL)
Saliva (n=17)	334	176	85.2 - 624
Urine (n=21)	280	171	85.2 - 628

Cell Culture Supernates -

Human Peripheral Blood Leukocytes (PBLs) were cultured in DMEM supplemented with 5% fetal calf serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernate were removed and assayed for levels of natural Clusterin.

Condition	Day 1 (ng/mL)	Day 6 (ng/mL)
Unstimulated	ND	ND
Stimulated	ND	ND

ND = Non-detectable

HepG2 cells were cultured in MEM (NEAA salts) supplemented with 10% fetal bovine serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. An aliquot of the cell culture supernate was and assayed for levels of natural Clusterin and measured 574 ng/mL.

HeLa cells were cultured in RPMI supplemented with 10% fetal bovine serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. An aliquot of the cell culture supernate was and assayed for levels of natural Clusterin and measured 863 ng/mL.

Human Umbilical Vein Endothelial Cells (HUVECs) were cultured in EGM-2 media and stimulated with recombinant human IL-1β for 24 hours. An aliquot of the cell culture supernate was removed, assayed for levels of natural Clusterin, and measured 17.3 ng/mL.

SPECIFICITY

This assay recognizes recombinant and natural human Clusterin. The factors listed below were prepared at 2 $\mu\text{g}/\text{mL}$ in Calibrator Diluent RD5T and assayed for cross-reactivity. Preparations of the following factors at 2 $\mu\text{g}/\text{mL}$ in a mid-range recombinant human Clusterin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

ApoA1
ApoA2
ApoB
ApoB100
ApoC1
ApoC2
ApoD
ApoE
ApoE3
ApoH
ApoM
CLUL-1

Recombinant mouse:

Clusterin

Recombinant rat:

Clusterin

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

	A	B	C	D	E	F	G	H
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								